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ON THE NATURE OF EARLY INTERMEDIATES of PHOTOSYNTHESIS.

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The photosynthetic phase that follows the photochemical act as well as the beginning of the process itself which includes the act is a very complicated problem. Its solution requires a wide scope of knowledge in many branches of science - from physics to biology and particularly in related fields of biology that are yet insufficiently investigated. While studying a concrete question in this problem it is necessary, therefore, to pay a special attention to the interrelation between all phenomena and processes that occur in the plant organism as a whole system. For the given biochemical investigation it means first of all to elucidate the question from the point of view of the interrelation between all aspects of metabolism. And the carbon pathway is the gold-bearing vein that has been successfully developed during the last ten years and gives splendid results in the development of biochemistry of photosynthesis.

The progress that resulted in the discovery of photosynthetic carbon-reduction cycle [1] has been made due to the works of Calvin and his colleagues and other investigators who employed the tracer method. The investigation of the sequence of $C^{14}O_2$ incoming to the compounds formed during the photosynthetic

process is the clue that makes it possible for the research workers to go along the labyrinth of reactions and offers a number of advantages over a variety of methods now employed.

The given paper mostly deals with the results recently obtained in our laboratory. They concern the elucidation of the sequence in the formation of CO_2 assimilation products under various conditions.

The methods were based on the following principles: to ensure normal physiological conditions for the object under experiment and possibly mild conditions at every stage of the material treatment. Since higher plants were usually employed in the experiments it was convenient to carry them out in the following way: The detached leaf was placed in a special network and run under mercury into a chamber with $C^{14}O_2$ in the air: / Fig. I /

Fig. I Apparatus for typical experiments

To gain a clear understanding of the sequence of the formation of photosynthetic product leaves in some tests were kept in the chamber about 1 sec. only and then they were immediately

placed into the second chamber with the equivalent amount of non-labeled CO_2 where they continued photosynthetizing in accordance with the test outline. Relying on the purpose of the experiment the material was fixed by plunging into boiling water, boiling alcohol, liquid air, cooled isopenthane, cooled acetone, etc. and then it was dried in vacuum at room temperature or by lyophilic method at the temperature below 0. The material ~~underwent~~ was subjected to further ~~different ways of~~ chromatographic analysis as it was earlier described /2/.

In our recent investigations we paid a particular attention to elucidation of the nature of initial products of CO_2 assimilation as it plays a key role in revealing the mechanism of photosynthesis. The attention paid to the problem has been intensified because, on the one hand, there appeared some new data concerning the properties of the presumed precursor of the first stable product of photosynthesis - phosphoglyceric acid /3,4/. It appeared that it can be isolated at the mild fixation of the material and at the usually employed hard fixation it transforms into phosphoglyceric acid. This cast doubt whether the phosphoglyceric acid itself is an artefact. On the other hand, new data were obtained relating to the methods of unstable-bounded CO_2 stabilization by hydroxylamin at the stage that is previous to the formation of photosynthetic products /5/. It offers new opportunities of studying its nature that has been considerably investigated in our laboratory earlier. /6,7/. Strong chemical reagents used as inhibitors of enzymatic reactions lead in some cases to the formation of another substance instead of phosphoglyceric acid.

However, basing on these observations we cannot draw a conclusion whether the phosphoglyceric acid is an intermediate in photosynthesis or not. To solve this problem means to show the sequence of the substance conversion under normal physiological conditions. To show this we tested , in the first place, leaves of different types of plants having in view to find such a leaf where enzyme systems and other biochemical, physiological and anatomic peculiarities create the conditions that disintegrate the phosphoglyceric acid precursor at a least degree. Secondly, we had to conduct the tests employing low temperatures and other mild conditions at every stage of the material treatment, including chromatography. We attained that in the following way. After exposure in the presence of $C^{14}O_2$ the leaves were quickly killed by cold while plunging into isopenthane cooled to -160°. Then they were ground with a mortar and pestle to a fine powder in liquid nitrogen; the obtained powder was mixed with a cooled solvent, in most cases - freezing ammonium. The latter has the advantage of immediate transferring the unstable product of ribulose diphosphate carboxylation - β-keto acid and its isomer γ-keto acid - into the corresponding salts which are more stable. Besides that, liquid ammonium dries the material and transfers to solution a number of products among which are amino acids, sugars, pigments and other substances. It seems probable that partial radioactivity transferring into the solution depends not only on $(NH_4)_2CO_3$. But phosphate esters that are of interest to us do not transit into the solution.

Extracted sediment is pumped off through the pressed paper filter. During the operation the temperature cannot rise higher than ammonium boiling point, i.e., -33°. The sediment is uniformly spread on the filter it is very convenient to keep it together with the filter in liquid ammonium. Small pieces of a definite size are cut out and pressed against the chromatographic paper with the help of glass clamps in the cold. Non-freezing mixtures of methanol-ammonium-water /80:II.5:27.5, buffer pH-3.7 butanol-ethanol-ammonium acetate /4:I:2/, alcohol-liquid ammonium, etc. served as cooled solvents.

Using such mild conditions to treat geranium leaves after their one-second exposure in the presence of $C^{14}O_2$ we detected on radiochromatograms at the beginning of diphosphate zone the radioactivity corresponding to the unstable product of CO_2 fixation on ribulose diphosphate that was absent from the same zone but treated with less carefulness. In the latter case radioactivity of phosphoglyceric acid increases. On the other hand, radioactivity decreases in the above mentioned zone and increases in phosphoglyceric acid if leaves are traced very quickly and photosynthesis proceeds for 2-3 seconds in the usual CO_2 with the following cold fixation and mild treatment of the material. The process was observed in geranium leaves. The results of the experiment can be seen in radioautogram / Fig. 2 /.

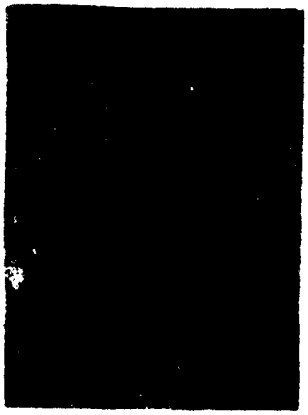

Fig.2 Radioautogram of photosynthetic products in geranium leaves
I. 1 sec. in $C^{14}O_2$, cold fixation
II. 1 sec. in $C^{14}O_2$ and 2 sec. in CO_2, cold fixation
III. 1 sec. in $C^{14}O_2$, hot fixation
IV. 1 sec. in $C^{14}O_2$ and 2 sec. in CO_2, hot fixation
a. Diphosphate zone
b. Phosphoglyceric acid zone

I 4

They prove that the product of CO_2 fixation on ribulose diphosphate is converted mainly through phosphoglyceric acid as a result of ~~a~~ normal photosynthesis in leaves. As it was previously shown by means of consistent exposure of leaves to labeled and non-labeled CO_2 the amount of tracer phosphoglyceric acid in kidney bean leaves decreased very quickly and only 5 seconds later it partially appeared again, more than half of radioactivity transferring from carboxyl group to other atoms /7/. This fact as well as the above mentioned data provides direct evidence of Calvin functioning photosynthetic luction cycle.

In 1951 /6/ we discovered the stage of unstable bound $C^{14}O_2$ that is prior to the formation of stable products in photosynthesis by higher plants leaves. At first we studied the relationship between the phenomenon and CO_2 uptake rate. We determined that under the conditions similar to the steady-state photosynthesis about 20 or even 50% of $C^{14}O_2$ of the whole tracer carbon absorbed during this space of time is unstable bound. The investigation of the nature of this phenomenon soon evidenced that it is of specific character and depends on a number of factors and resembles ~~general~~ chemical absorption in general /7/. Unstable bound $C^{14}O_2$ cannot be removed by vacuum but can be displaced by excessCO_2. Light increases the ability of CO_2 unstable binding in leaves. In the dark under normal physiological conditions the above mentioned ability decreases but very slowly, much slower than acceptor ribulose diphosphate disappears. Various poisons and factors reducing leaf vitality in general eventually decrease CO_2 unstable binding. Killed leaves have no ability of quick labile binding

of CO_2 at such a scale. Some enzymatic inhibitors act more specifically. CO_2 unstable binding reduces greatly (CN)$_2$; phenylurethane vapour is less effective. PCMB (p-chlormercurybensoat) and hydroxylamin infiltrated in leaves reduce CO_2 adsorption as well. Ethiolized leaves possess a considerably less ability of CO_2 quick unstable binding but they cannot be compared with the blank because of the conditions.

These data are given in Table I.

The data were obtained in the following way. Leaves corresponding to certain conditions of the experiment after exposure in chamber were fixed instantly cold and ground to powder under liquid nitrogen. The suspension was run into the cooled alcohol with acetic acid pH~5 to remove CO_2 solved partially and buffered. Some parts of the suspension were placed in cooled vessels of Thunberg tubing type. Pear-shaped tubes were filled with alcohol solution $Ba(OH)_2$ + $BaCl_2$. Several vessels that served as a blank were heated and suspension was boiled during 5 min, then Ba-solution added. From these vessels diluted tests were taken for counting. Other vessels were immediately closed, suspension was mixed with Ba-solution and then gradually heated. In these vessels general radioactivity was determined. The difference between general and stable radioactivity was in agreement with the amount of unstable bound carbon.

Table I.

$C^{14}O_2$ unstable binding by leaves under various conditions

No.	Objects and conditions	Time of exposure	Counts per min. / g of wet weight x 10^{-3}		
			Total uptake	Stable bound $C^{14}O_2$	Unstable bound $C^{14}O_2$
I.	Normal kidney bean leaves light	I sec.	3I.6	23.5	8I
2.	light	5 "	I43.3	I22.7	20.6
3.	dark	I "	6.2	0.8	5.4
4.	dark 2 hours	I "	2,7	0.3	2.4
5.	Leaves killed by vapour at I00°	I "	0.2I	0.0I	0.2
6.	30 min. in $(CN)_2 I0^{-2}$M light	I "	6.3	4.0	2.3
7.	40 min. in phenyl-urethane vapour light	I "	II.9	7.2	4.7
8.	40 min. in I% NH_3 /light	I "	I6.4	I0.5	5.9
9.	Solution of PCMB /infiltrated/	I "	9.0	4.8	4.2
I0.	Solution of hydroxylamin	I "	I0.5	7.8	2.7
II.	2 hours in light	I "	27.0	I9.3	7.7
I2.	2 hours in dark	I "	I.I9	0.09	I.I.

Metzner and Calvin /5/ are known to discover and deeply investigate the ability of CO_2 unstable binding by photosynthetizing algae. They detected hydroxylamin stabilizing unstable bound CO_2. The observation can throw some light on the nature of the formed compounds. The authors suggested that CO_2 unstable bound with some groups is activated, its assimilation rate being accelerated to compensate insufficient activity of the enzyme that carboxylates ribulose diphosphate. There is no ground to seek for the reason of the slow rate reaction of ribulose diphosphate carboxylation in a different form of the substrate and not in a different form of the enzyme itself studied only in vitro. However, the presence of $[CO_2]$ active form is very probable. This is confirmed by Ruttner's observations /9/ who showed that aqueous mosses assimilate free CO_2 while many algae use HCO_3^- ions.

To elucidate CO_2 assimilation process from the point of view of the interrelation between different aspects of metabolism means to study CO_2 products in various types of organisms under diverse conditions. Comparative investigations of photosynthetic products in dynamics in 20 types of algae Scenedesmus and bacteria Rodopsendomonas palustris led to the conclusion /IO, II/ that the principle stages of carbon pathway in photosynthesis are uniform though metabolism peculiarities and immediate dependence on the conditions reveal themselves at the very beginning. Even the first substances to be labeled are transformed in a different way relying on the

type of metabolism. For instance, it is proved that phosphoglyceric acid (and partially, its precursors) transforms with reduction, oxidation, dephosphorylation as a minimum. But the transformation proceeds chiefly as a photosynthetic reduction cycle. Dependence on the conditions shows itself very quickly. It refers especially to light intensity, temperature and mineral feeding /I2/. E.g. oxidized or reduced form of nitrogen infiltrated in leaves effects the composition of photosynthetic products even at a short exposure in a different way /I3/. Temperature rise, weak light and many factors that are unfavourable for photosynthesis in general lead, first of all, to the relative fall of reduced phosphoglyceric acid amount. At the same time relative radioactivity in products of various reactions in carboxylation increases. This does not mean however the increase of reaction intensity.

Different reactions of carboxylation in the synthesis of plastic substance in a plant organism are of great importance. Though many of them can be inhibited in the light as a result of their competition for CO_2 intensity of others increases within certain limits. E.g., the intensity of reducing carboxylation of pyruvate acid rises as a result of the substance accumulated during the photosynthetic process /I,7/. Calvin noticed citrulline synthesis accelerating in the light through ornithine cycle. In our laboratory a new type of reaction of CO_2 fixation was discovered. The reaction proceeds at a small rate simultaneously with CO_2 fixation on ribulose diphosphate and leads to synthesis of the substance resembling a polyphenol carboxylation product /II/. The synthesis of the substance

moving on chromatograms with the phenol group was observed in all experiments but it could be found in some plants only: kidney bean, pea, tobacco, begonia /Fig. 3/.

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Fig. 3. Radioautogram of photostnthetic products of kidney bean leaves. Exposure IO sec., t ~ 7-8° C
I. Presumed product of polyphenol carboxylation

Comparing photosynthetic and chemosynthetic pathways of CO_2 assimilation we obtained the following data. Chemosynthetizing hydrogen bacteria besides carbon pathway analogous to photosynthetic through ribulose diphosphate and phosphoglyceric acid have another carbon pathway coupled with an early formation of the substance of the principle character. It seems likely that the formation of these substances though at a small degree proceeds in plants as well.

During CO_2 dark fixation by plant leaves some products are known to be formed mainly as a result of carboxylation reactions. We obtained some data /I4/ showing the presence of several independent ways of CO_2 dark uptake that lead, mainly, to the synthesis and accumulation of substances coupled with respiratory cycle.

Thus, the main pathway of CO_2 assimilation through the photosynthetic reduction cycle developing in chloroplasts is coupled with synthesis of

many plastic substances related to another very important vital process of plants - respiration. Respiratory reactions according to the recent data of James predominate in cytoplasm. In our laboratory radioautographs were obtained that reveal localization of photosynthetic initial products on the surface of chloroplasts in leaves "mnium" consisting of a single layer of cells. This kind of localization of photosynthetic initial products promoted their further transformation in the photosynthetic and in the respiratory process as well.and strengthens the interrelation between these two processes.

Thus, various reactions of CO_2 assimilation are incorporated in the uniform process of metabolism which is the basis of all vital phenomena.

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